Journal of Chromatography, 363 (1986) 438–443
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 822

Note

Application of ion-exchange high-performance liquid chromatography in the purification of 5S rRNAs suitable for sequence analysis

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(First received April 7th, 1986; revised manuscript received May 23rd, 1986)

A simple, dependable size-exclusion or ion-exchange method for the liquid chromatographic separation of tRNAs and 5S rRNA is not available. Indeed, the method of choice for purification of small RNA species consists of electrophoretic separation on denaturing polyacrylamide gels. Methods for purifying small oligoribonucleotides using either conventional[1,2] or thiol-soluble[3] polyacrylamide gels are well developed. Although these methods are dependable and result in minimal loss during recovery of samples from the gel, they nevertheless fall short of high-performance liquid chromatographic (HPLC) methods in terms of speed, labor, reduced risk of error and ease of sample recovery.

Even though tRNA and 5S rRNA molecules are significantly different in length, chromatographic separation of the two molecules is not necessarily easy. tRNAs comprise a heterogenous collection of specific amino acid acceptors, the average length of which is *ca.* 75 bases; 5S rRNA is virtually homogenous, comprising 116 to 122 bases in prokaryotes, depending upon species. Despite a significant difference in length, tRNAs and 5S rRNAs share remarkable similarities in secondary and tertiary structure (Fig. 1), possibly attributable to having derived from a common origin[4].

In this paper we describe a rapid and reliable HPLC method for purifying of 5S rRNA from biological samples, with sufficient homogeneity of the preparations for sequence analysis.

EXPERIMENTAL

Samples

An aqueous nucleic acid solution, rich in tRNA, mRNA, 5S rRNA and 16S rRNA, was prepared from bacterial cell paste (approximately 1 g wet weight) as follows. Cells were lysed using a freeze–thaw technique[7] and phenol extracted[8] with a solution composed of 89% (w/v) phenol, 0.1% (w/v) 8-hydroxyquinoline in 50 m*M*

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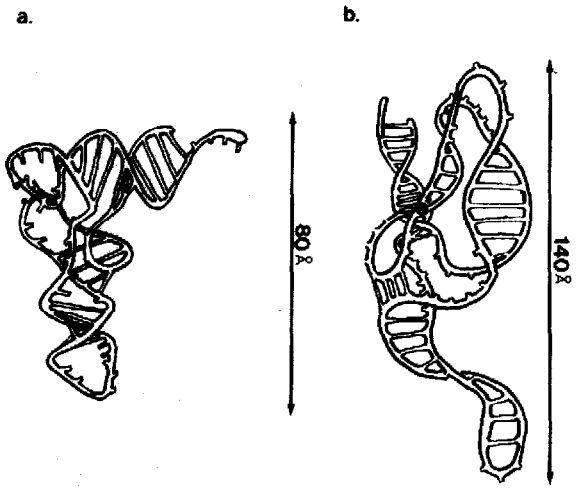

Fig. 1. Predicted tertiary structures of (a) tRNA (adapted from ref. 5) and (b) 5S rRNA (adapted from ref. 6).

Tris borate EDTA (TBE), pH 8.3. After centrifugation for 10 min, at 12 000 *g*, the aqueous (upper) phase was collected and precipitated in two parts cold absolute ethanol. The ethanolic precipitates were chilled on crushed ice for 10 min[9] and collected by centrifugation (10 min at 12 000 *g*). This "total nucleic acid" fraction was then dried using a vacuum line.

Enrichment for RNAs by ion-exchange chromatography

The dried nucleic acid pellet was resuspended in 10 ml of 50 m*M* TBE (pH 8.3) and adsorbed onto DEAE-cellulose (Cellex D; Bio-Rad, Richmond, CA, U.S.A.), poured to a bed height of 4–5 cm in a 150 × 9 mm I.D. disposable glass column (Chromoflex; Kontes Scientific Glassware, Vineland, NJ, U.S.A.). The column was washed with two volumes of 50 m*M* TBE, pH 8.3, followed by a two-column volume rinse with 0.2 *M* sodium chloride in 50 m*M* TBE. The "small RNA" fraction was eluted with 0.5 *M* sodium chloride and 7 *M* urea in 50 m*M* TBE. The RNA was precipitated by addition of two volumes of cold absolute ethanol and chilling on crushed ice for 10 min. The precipitated RNA fraction was collected and dried as described above.

Separation

The precipitated "small RNA" fraction was dissolved in 200–500 μl of sterile distilled water and injected, in 200-μl aliquots, onto a Spherogel DEAE TSK-IEX 5PW ion-exchange column (75 × 7.5 mm I.D.) (Beckman, Berkeley, CA, U.S.A.) in the presence of 200 m*M* potassium chloride, 5 *M* urea in 20 m*M* sodium phosphate, pH 6.9, and fractionated using a two-step linear gradient. A Beckman Model 334 HPLC system with a Model 165 detector was used throughout. The gradient program used on the HPLC controller is given in Table I.

TABLE I

HPLC CONTROLLER PROGRAM LISTING

*Time** *(min)*	*Function*	*Value*	*Duration*** *(min)*
0:00	Percent B	20	
0:00	Flow-rate (ml/min)	1	
0:00	Chart-speed (cm/min)	0.1	
4:00	Percent B	30	10
14:00	Percent B	60	60

* Execution time from start of program.

** length of time allowed for the completion of a program instruction. Example: in the last line, percent B would increase from 30% (previous instruction) to 60% over a period of 60 min.

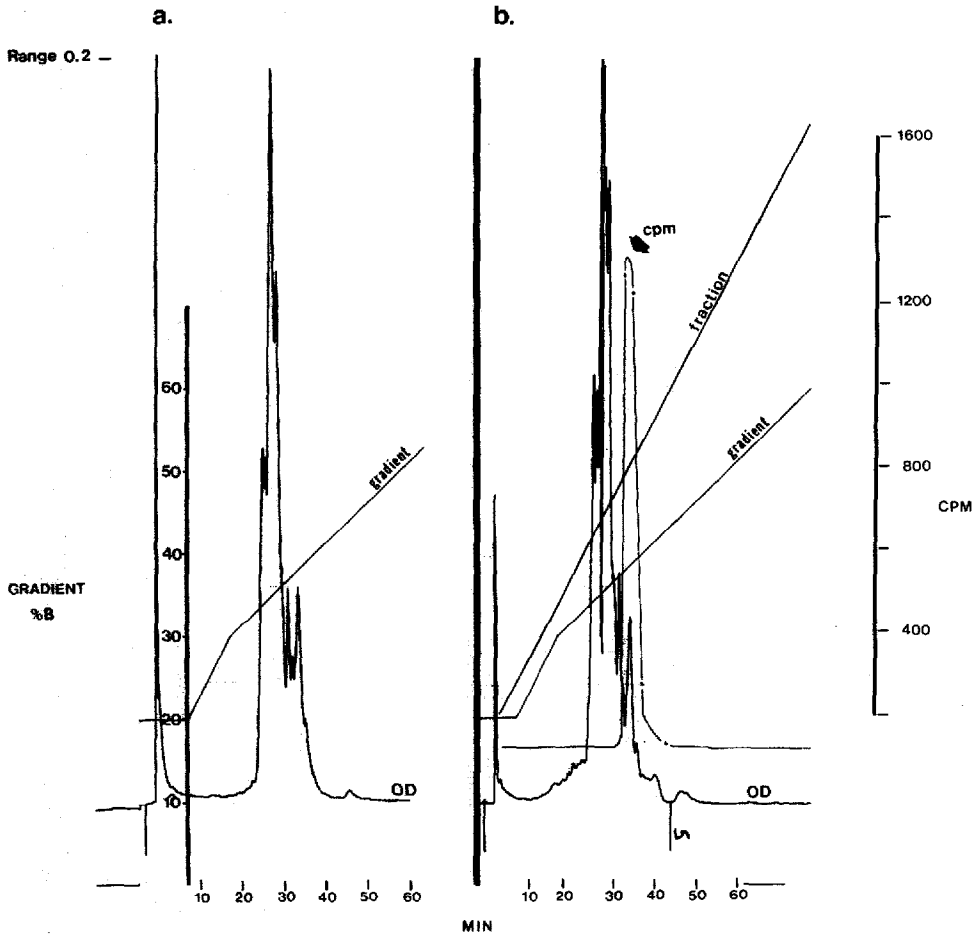

Fig. 2. HPLC Chromatograms of an admixture of purified yeast phenylalanine tRNA and purified *Escherichia coli* 5S rRNA. Column, Beckman Spherogel TSK-IEX DEAE-5PW; mobile phase 200–600 m*M* potassium chloride in 5 *M* urea, 20 m*M* sodium phosphate buffer, pH 6.9, in a two-step linear gradient; flow-rate, 1 ml/min; chart speed, 1 mm/min; range, 0.2. (a) Optical density profile. (b) Repeat of (a) in which 5S rRNA was identified in the elution order by spiking the admixture with *E. coli* ^{32}P-labeled 5S rRNA.

Polyacrylamide gel electrophoresis

Dried RNA samples were suspended in a tracking dye loading buffer consisting of 0.05% (w/v) xylene cyanol, 0.05% bromophenol blue, 10 *M* urea, 20 m*M* TBE (pH 8.3) and electrophoresed for 90 min at 40 V/cm and stained with ethidium bromide. Presumptive identifications of tRNA and 5S rRNA were made on a basis of banding pattern and location with respect to tracking dyes.

RESULTS AND DISCUSSION

To test for the separation of tRNA and 5S rRNA, a mixture of purified tRNA

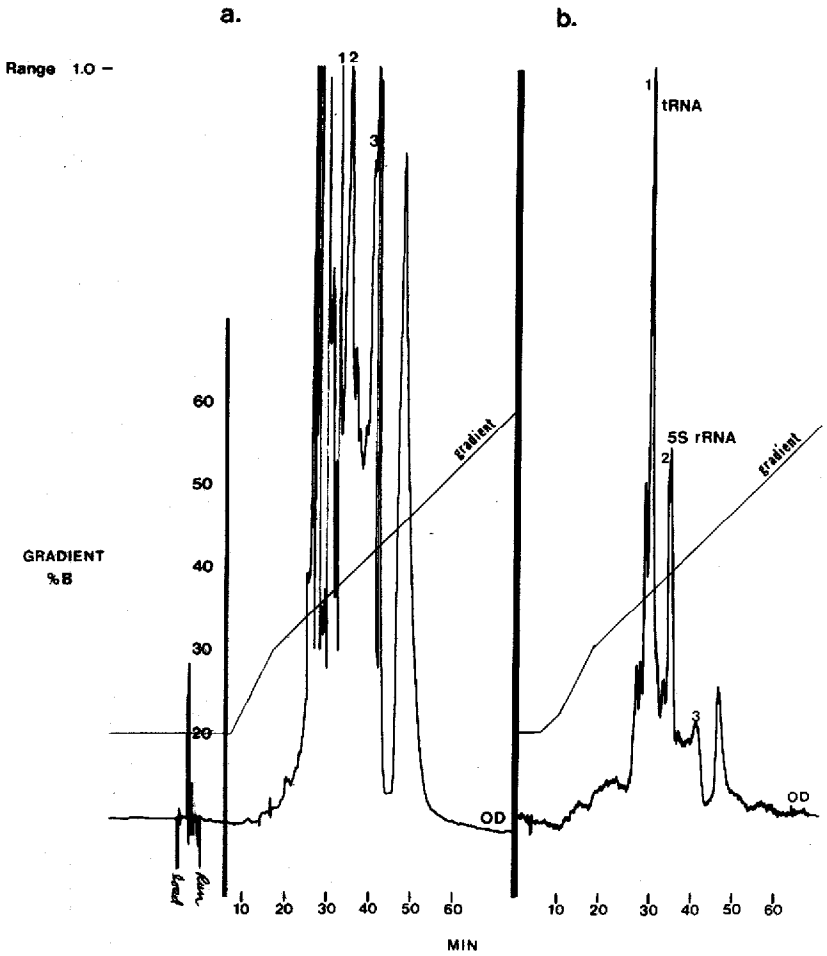

Fig. 3. HPLC chromatograms of the "small RNA" fraction (see text for discussion). (a) Chromatogram of the crude RNA fraction prepared from the bacterial cell lysate. Eluates corresponding to peaks 1–3, selected on a basis of their elution order and positions relative to the buffer gradient, were collected and ethanol precipitated. Aliquots of RNAs from each peak were electrophoresed on polyacrylamide for verification (see Fig. 4). An aliquot of peak 2 was re-injected onto the HPLC column. (b) Chromatogram of the RNA collected from peak 2. Chromatographic conditions were as described in Fig. 2.

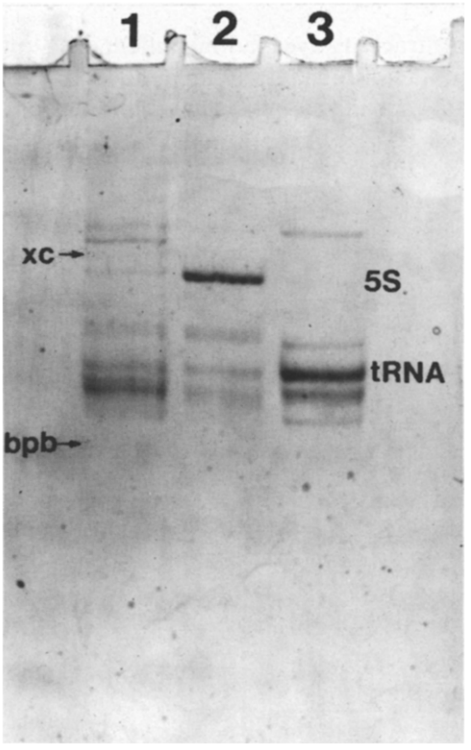

Fig. 4. Ethidium bromide-stained 5% polyacrylamide gel after electrophoretic separation of ethanol precipitates corresponding to peaks 1–3 (see Fig. 3). Lanes 1 and 3 correspond to peaks 1 and 3 in the chromatogram. The pattern and location of bands in lanes 1 and 3 are identical to those expected of tRNAs. The prominent band in lane 2 (corresponding to peak 2) is consistent with the expected position of 5S rRNA (see text for discussion). Locations of tracking dyes bromophenol blue (bpb) and xylene cyanol (xc) are indicated.

and unlabelled 5S tRNA was loaded onto the HPLC column and a two-step linear gradient was run (see Experimental section). Results of this separation are shown in Fig. 2a. The chromatography was then duplicated using ^{32}P-labeled 5S rRNA (Fig. 2b). It was observed that 5S rRNA was successfully separated from tRNAs in biological samples by a two-step process involving enrichment for small oligoribonucleotides, using conventional DEAE-cellulose ion-exchange column chromatography, followed by separation by ion-exchange HPLC. In this procedure, small fragments, monomers and very small oligomers (<40 bases) are eluted from the DEAE-cellulose with 0.2 *M* sodium chloride in 50 m*M* TBE, after which the fraction containing tRNA, 5S rRNA, mRNA and 16S rRNA was eluted with 0.5 *M* sodium chloride, 7 *M* urea in 50 m*M* TBE. Very large oligonucleotides, plasmids and chromosomal DNA remain on the DEAE-cellulose under these conditions. We have concluded that further separation of tRNA and 5S rRNA is not feasible at this step since these RNAs are co-eluted from DEAE-cellulose regardless of the solvent conditions. The "small RNA" fraction, *i.e.*, tRNA through 16S rRNA, was precipitated

in two volumes cold absolute ethanol, collected and dried as above. The dried pellet was suspended in 100 μl of TBE and injected onto the HPLC column. The chromatogram (Fig. 3a) shows several peaks in the vicinity of the expected 5S rRNA peak. Of considerable interest were those RNAs which were eluted from (i) 32% to 38% B, (ii) 38% to 39% B, and (iii) 41% to 43% B.

Samples were collected corresponding to peaks numbered 1, 2 and 3 in the chromatogram (Fig. 3) and the nucleic acids precipitated. An aliquot corresponding to peak 2 was re-injected onto the HPLC column (Fig. 3b) to evaluate chromatographic purity. In addition, aliquots corresponding to peaks 1 through 3 were collected and electrophoresed on 5% polyacrylamide to identify presumptively the RNA species, based on characteristic mobilities. Bands corresponding to peaks 1 and 3 migrated as tRNAs, while peak 2 migrated as 5S rRNA (Fig. 4).

To identify peak 2 as 5S rRNA, nucleic acid was collected, endlabeled with [^{32}P]ATP, using the method of Richardson[10], and sequenced enzymatically[11]. The results of the sequence analysis indicated that the 5S rRNA band was homogenous and clearly identifiable as the sequence of 5S rRNA.

CONCLUSIONS

Despite the tendency of tRNAs and 5S rRNAs to be co-eluted during chromatographic separations of crude RNA solutions prepared from cell lysates, adequate separation is possible by ion-exchange HPLC to allow sequence analysis of 5S rRNA collected directly from the eluate. This represents a considerable savings in time, labor and materials, and prevents loss of sample, compared with purification using polyacrylamide gels.

ACKNOWLEDGEMENTS

We wish to acknowledge D. B. Roszak for helpful discussion. Support for this research was provided by NSF Grant BSR 84-01397, Office of Naval Research contract N000-14-81-K0638, EPA contract CR812246-01-0, and The University of Maryland Center of Marine Biotechnology.

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